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(57) Abstract

A reduced-toxicity formulation of carotenoids is disclosed which is stable in an aqueous environment. The formulation includes a carotenoid, lipid carrier particles (such as liposomes), and an intercalation promoter agent (such as a triglyceride), which causes the carotenoid to be substantially uniformly distributed with the lipid in the lipid carrier particles. The molar ratio of carotenoid to lipid is greater than about 1:10. Also disclosed is a method of inhibiting the growth of cancer cells, which comprises administering to a living subject a therapeutically effective amount of a composition as described above.

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FORMULATION AND USE OF CAROTENOIDS IN TREATMENT OF CANCER

This application is a continuation-in-part of U.S. serial no. 588,143, filed on September 25, 1990, which is a divisional of U.S. serial no. 152,183, filed on February 4, 1988, now abandoned. The two above-identified applications are incorporated here by reference.

The present invention relates to therapeutic compositions of carotenoids encapsulated in liposomes or other lipid carrier particles.

It has been known for more than 50 years that retinoids, the family of molecules comprising both the natural and synthetic analogues of retinol (vitamin A), are potent agents for control of both cellular differentiation and cellular proliferation (Wolbach et al., J. Exp. Med., 42:753-777, 1925). Several studies have shown that retinoids can suppress the process of carcinogenesis in vivo in experimental animals (for reviews, see e.g., Bollag, Cancer Chemother. Pharmacol., 3:207-215, 1979, and Sporn et al., In Zedeck et al. (eds.), Inhibition of Tumor induction and development, pp. 71-100. New York: Plenum Publishing Corp., 1981). These results are now the basis of current attempts to use retinoids for cancer prevention in humans. Furthermore, there is extensive evidence which suggests that retinoids can suppress the development of malignant phenotype in vitro (for review, see e.g., Bertram et al., In: M.S. Arnott et al., (eds.), Molecular

interactions of nutrition and cancer, pp 315-335. New York, Raven Press, 1982; Lotan et al., The modulation and mediation of cancer by vitamins, pp 211-223. Basel: S. Karger AG, 1983) thus suggesting a potential use of retinoids in cancer prevention. Also, recently it has been shown that retinoids can exert effects on certain fully transformed, invasive, neoplastic cells leading in certain instances to a suppression of proliferation (Lotan, Biochim. Biophys. Acta, 605:33-91, 1980) and in other instances to terminal differentiation of these cells, resulting in a more benign, non-neoplastic phenotype (see e.g., Brietman et al., Proc. Natl. Acad. Sci. U.S.A., 77:2936-2940, 1980).

Retinoids have also been shown to be effective in the treatment of cystic acne (see e.g., Peck, et al., New Engl. J. Med., 300:329-333, 1979). In addition to cystic acne, retinoid therapy has been shown to be effective in gram-negative folliculitis, acne fulminans, acne conglobata, hidradenitis suppuritiva, dissecting cellulitis of the scalp, and acne rosacea (see e.g., Plewig et al., J. Am. Acad. Dermatol., 6:766-785, 1982).

However, due to highly toxic side effects of naturally occurring forms of vitamin A (hypervitaminosis A) at therapeutic dose level, clinical use of retinoids has been limited (Kamm et al., In: The Retinoids. Sporn et al., (eds.), Academic Press, N.Y., pp 228-326, 1984; Lippman et al., Cancer Treatment Reports, 71:493-515, 1987). In free form, the retinoids may have access to the surrounding normal tissues which might be the basis of their profound toxicity to liver, central nervous system, and skeletal tissue.

Therefore, one potential method to reduce the toxicity associated with retinoid administration would be the use of

a drug delivery system. The liposomal format is a useful one for controlling the topography of drug distribution in vivo. This, in essence, involves attaining a high concentration and/or long duration of drug action at a target (e.g. a tumor) site where beneficial effects may occur, while maintaining a low concentration and/or reduced duration at other sites where adverse side effects may occur (Juliano, et al., In: Drug Delivery Systems, Juliano ed., Oxford Press, N.Y., pp 189-230, 1980). Liposome-encapsulation of drug may be expected to impact upon all the problems of controlled drug delivery since encapsulation radically alters the pharmacokinetics, distribution and metabolism of drugs.

There are additional difficulties in using a liposomal formulation of a retinoid for therapeutic purposes. For example, it is often desirable to store the composition in the form of a preliposomal powder, but many prior formulations are not satisfactory for such use, because they either contain an inadequate amount of retinoid, or they generate undesirable liposomes when they are reconstituted in aqueous solution.

For compositions that are to be administered intravenously, typically the composition must provide at least about 100 mg of the active ingredient in a single container; if it contains a lesser amount of the active ingredient, an impractically large number of vials will be needed for dosing a single patient.

Typically a vial having a volume of 120 cc is the largest that can be accommodated in a commercial freeze drier, and 50 cc is the maximum volume of liquid that can be filled in such a vial. If more than 1 g of lipids are included in 50 cc of liquid volume, the resulting liposomes after reconstitution have a size distribution which is not

acceptable for parenteral administration. This is because the packing of the lipids during lyophilization is affected by the concentration of the lipids in the solution. Thus, the concentration of lipids in the solution must be limited. However, when this is done in previously-known liposomal retinoid formulations, the retinoid tends to crystallize, and separate from the liposomes shortly after reconstitution.

In order to both limit the concentration of lipids and supply a sufficient amount of retinoid, it is necessary to provide a molar ratio of retinoid to lipid greater than about 1 to 10. Previously known formulations have not had, and are believed not to be capable of having such a high packing of retinoid in the liposomes. Therefore, a need exists for improved compositions and methods which will minimize or eliminate the problems of the prior art.

The present invention relates to therapeutically useful, reduced toxicity compositions of carotenoids. The compositions comprise a carotenoid, lipid carrier particles, and an intercalation promoter agent. "Carotenoid" is used here to include retinoids, pro-retinoids, carotenes, xanthophylls, and analogs thereof. A preferred example is all-trans retinoic acid. The carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles. More particularly, the carotenoid is substantially uniformly distributed in an intercalated position throughout a hydrophobic portion of the lipid carrier particles, as opposed to the aqueous phase. "Substantially uniformly distributed" means that at least 50% of the lipid carrier particles will contain carotenoid in a molar ratio between about 5:85 carotenoid:lipid and about 15:70. Preferably at least 75% of all lipid carrier particles will contain such a ratio of the active ingredient.

The composition is stable in an aqueous environment. In this context, "stable in an aqueous environment" means that the composition (1) will not exhibit any therapeutically significant degradation over a period of at least 24 hours, (2) will not exhibit a substantial degree of fusions of liposomes over that same period, and (3) will not exhibit substantial redistribution of the carotenoid over that same period, including no substantial movement of the drug into the aqueous phase of a liposome, and no substantial state change into a crystalline form.

The molar ratio of carotenoid to lipid in the lipid carrier particles is greater than about 1:10, and is most preferably at least about 15:85. The intercalation promoter agent preferably comprises at least about 15% by weight of the composition, and can suitably be, for example, a triglyceride.

"Lipid carrier particles" is used here to include liposomes, having a bilayer structure formed of one or more lipids having polar heads and nonpolar tails, as well as micelles, amorphous particulates of lipid, and other lipid emulsion state entities. When the particles are liposomes, suitable forms include multilamellar liposomes.

The present invention also relates to a pharmaceutical unit dosage formulation of a carotenoid, which comprises a carotenoid, lipid carrier particles, an intercalation promoter agent, and a pharmaceutically acceptable carrier. As stated above, the carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles, and the composition is stable in an aqueous environment.

In another aspect, the invention relates to a method of inhibiting the growth of cancer cells, in which a therapeutically effective amount of a carotenoid

composition is administered to a living subject. The carotenoid composition can be as described above. The composition is preferably administered to the subject in a maintained molar ratio between about 5:85 carotenoid:lipid and about 15:70. "Maintained" in this context means that the stated ratio of drug to lipid lasts for at least 24 hours.

The present invention provides the therapeutic benefits of the carotenoid, while substantially reducing the undesirable toxicity of the composition, as compared to the free drug. For example, encapsulation of retinoic acid in liposomes results in a decrease of at least 15-fold in toxicity as compared to the free drug.

Further, the presence of the intercalation promoter agent permits the ratio of active ingredient to lipid to be increased above what has been previously known, and thus makes such formulations useful in a practical sense for lyophilization into a powder, and subsequent reconstitution into solution which can be administered parenterally to a patient. Without wishing to be bound by any particular theory, it is believed that the intercalation promoter agent overcomes steric hindrance that otherwise limits the amount of carotenoid that be incorporated in, for example, a liposome.

The encapsulation of carotenoids within, e.g., liposomes, permits their direct delivery to intracellular sites and thus circumvents the requirement for cell surface receptors. This may be of particular significance, for example, in therapy of tumors which lack the cell surface receptors for serum retinol binding protein but possess intracellular receptors for retinoic acid.

Compositions of the present invention are also substantially improved over prior liposomal retinoid formulations in terms of uniformity of drug distribution. Prior compositions often had substantial percentages of liposomes which contained essentially no drug. In the present invention, at least 50% and preferably at least 75% of all liposomes in the composition contain drug with the range specified above.

Figure 1 shows a time profile of liposomal retinoic acid (L-RA) stability in the presence (○) and absence (O) of serum.

Figure 2 shows human red blood cell (RBC) lysis as a function of time with RA (○) and L-RA (△).

Figure 3 shows RBC lysis as a function of retinoic acid (RA) concentration (○) and L-RA concentration (△).

Figure 4 shows the inhibition of THP-1 cell growth as a function of RA concentration (○), L-RA concentration (O) or empty liposome concentration (△).

Figure 5 shows the induction of transglutaminase (TGase) in human monocytic THP-1 cells as a function of treatment with RA or L-RA.

Figure 6 shows the inhibition of human histiocytic U-937 cell growth as a function of RA concentration (○), L-RA concentration (O) and empty liposome concentration (△).

Figure 7 shows the time course of accumulation of tissue TGase activity in cultured human peripheral blood monocytes (HPBM). HPBM were fractionated into small (O) and large (○) subpopulations by centrifugal elutriation, and they were cultured in 35-mm-well tissue culture plates

as described in Materials and Methods. At the indicated time points the cells were washed, sonicated, and assayed for TGase activity. Values are the means of six determinations from two dishes.

Figure 8 shows dose-dependent effects of recombinant interferon-gamma (rIFN-g) on induction of tissue TGase activity in HPBM subpopulations. Small (O) and large (∘) monocytes were cultured in serum containing medium alone or medium containing increasing concentrations of rIFN-g. After 72 hr, the cells were harvested and the cell lysates assayed for tissue TGase activity. The results shown represent mean ± SD of three determinations from an individual donor.

Figure 9 shows effects of retinol (ROH) and RA on induction of tissue TGase activity in cultured HPBM. Cells were cultured in the presence of 5% human AB serum and the absence (O) or presence of 500 nM ROH (△) or RA (∘) for varying periods of time. At the end of each time point, the cells were harvested and assayed for enzyme activity. Values shown are the means ± SD of six determinations from two independent experiments. Inset, dose-response curve for tissue TGase induction by ROH (△) and RA (∘) in HPBM after 72-hr culture.

Figure 10 shows effects of free- and liposome-encapsulated RA on induction of tissue TGase in HPBM. A: The cells were cultured in tissue culture dishes in presence of serum-containing medium alone (△) 500 nm liposomal RA (∘), or medium containing 500 nM free-RA (△), or "empty liposomes" (O) for indicated periods of time. Both the liposomal RA and "empty liposomes" contained 200 ug/ml lipid. At the end of each time point, the cultures were washed and cell lysates assayed for TGase activity. Values shown are the mean ± SD of six determinations from

two independent experiments. B: Western-blot analysis of the levels of tissue TGase in freshly isolated HPBM (lane 1) and in HPBM cultured for 72 hr in the presence of serum-containing medium alone (lane 2), in medium containing 500 nM free RA (lane 3), 500 nM liposomal RA (lane 4), or "empty liposomes" (lane 5). Cell lysates containing 25 ug of protein were subjected to Western-blot analysis as described in Materials and Methods.

Figure 11 shows effect of free and liposome-encapsulated ROH on induction of tissue TGase in HPBM. A: HPBM monolayers were cultured in serum-containing medium alone (▵) or medium containing 1 uM of free- (○) or liposomal-ROH (▵) for 72 hr. Then the cultures were washed and the cell lysates assayed for enzyme activity as described in Materials and Methods. B: Western-blot analysis of tissue TGase levels in freshly isolated HPBM (lane 1) and in HPBM cultured for 72 hr in the presence of serum-containing medium alone (lane 2), in medium containing 1 uM of free ROH (lane 3), or liposome-encapsulated ROH (lane 4) as described in Materials and Methods. Twenty-five micrograms of cell protein was loaded onto each lane.

Suitable therapeutic carotenoids for encapsulation in accordance with the present invention include various retinoids. Trans-retinoic acid and all-trans-retinol are preferred. Other retinoids that are believed suitable include: retinoic acid methyl ester, retinoic acid ethyl ester, phenyl analog of retinoic acid, etretinate, retinol, retinyl acetate, retinaldehyde, all-trans-retinoic acid, and 13-cis-retinoic acid.

Lipid carrier particles, such as liposomes, can be formed by methods that are well known in this field. Suitable phospholipid compounds include phosphatidyl

choline, phosphatidic acid, phosphatidyl serine, sphingolipids, sphingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides, phosphatides, sterols, and the like. More particularly, the phospholipids which can be used include dimyristoyl phosphatidyl choline, egg phosphatidyl choline, dilauryloyl phosphatidyl choline, dipalmitoyl phosphatidyl choline, distearoyl phosphatidyl choline, 1-myristoyl-2-palmitoyl phosphatidyl choline, 1-palmitoyl-2-myristoyl phosphatidyl choline, 1-palmitoyl-2-stearoyl phosphatidyl choline, 1-stearoyl-2-palmitoyl phosphatidyl choline, dioleoyl phosphatidyl choline, dimyristoyl phosphatidic acid, dipalmitoyl phosphatidic acid, dimyristoyl phosphatidyl ethanolamine, dipalmitoyl phosphatidyl ethanolamine, dimyristoyl phosphatidyl serine, dipalmitoyl phosphatidyl serine, brain phosphatidyl serine, brain sphingomyelin, dipalmitoyl sphingomyelin, and distearoyl sphingomyelin.

Phosphatidyl glycerol, more particularly dimyristoyl phosphatidyl glycerol (DMPG), is not preferred for use in the present invention. In the carotenoid compositions of the present invention, the presence of DMPG correlates with the appearance of amorphous structures of anomalous size, which are believed to render the composition much less suitable for intravenous administration. When DMPG is omitted, the amorphous structures are not observed. The undesirable effects that are apparently caused by the presence of DMPG may result from the fact that DMPG has a negative charge, which may interact with the carboxylate of the carotenoid.

In addition, other lipids such as steroids and cholesterol may be intermixed with the phospholipid components to confer certain desired and known properties on the resultant liposomes. Further, synthetic phospholipids containing either altered aliphatic portions,

such as hydroxyl groups, branched carbon chains, cyclo derivatives, aromatic derivatives, ethers, amides, polyunsaturated derivatives, halogenated derivatives, or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quaternary amine, sulfate, sulfonate, carboxy, amine, sulfhydryl, imidazole groups and combinations of such groups, can be either substituted or intermixed with the phospholipids, and others known to those skilled in the art.

A suitable intercalation promoter agent will permit the high molar ratio of carotenoid to lipid that is desired for the present invention, without substantial crystallization from the liposomes after they are reconstituted in aqueous solution, as can be observed by microscopic analysis, separation techniques based on buoyant density, or other techniques well known to those skilled in the art. Triglycerides are preferred intercalation promoter agents, with soybean oil as one specific example. Other suitable agents include sterols, such as cholesterol, fatty alcohols, fatty acids, fatty acids esterified to a number of moieties, such as polysorbate, propylene glycol, mono- and diglycerides, and polymers such as polyvinyl alcohols.

Prior to lyophilization, the carotenoid, lipids, and intercalation promoter agent can be dissolved in an organic solvent, such as t-butanol. Lyophilization to form a preliposomal powder can be performed using commercial apparatus which is known to persons skilled in this field. After lyophilization, the powder can be reconstituted as, e.g., liposomes, by adding a pharmaceutically acceptable carrier, such as sterile water, saline solution, or dextrose solution, with agitation, and optionally with the application of heat.

A preferred formulation, which can be dissolved in 45 ml of t-butanol, is as follows:

component	mg	millimoles	mole %	wt %
DMPC	850	1.28	72	77
soybean oil	150	0.17	9	14
tretinoin	100	0.33	19	9

A composition of the present invention is preferably administered to a patient parenterally, for example by intravenous, intraarterial, intramuscular, intralymphatic, intraperitoneal, subcutaneous, intrapleural, or intrathecal injection. Administration could also be by topical application or oral dosage. Preferred dosages are between 40-200 mg/m^2. The dosage is preferably repeated on a timed schedule until tumor regression or disappearance has been achieved, and may be in conjunction with other forms of tumor therapy such as surgery, radiation, or chemotherapy with other agents.

The present invention is useful in the treatment of cancer, including the following specific examples: hematologic malignancies such as leukemia and lymphoma, carcinomas such as breast, lung, and colon, and sarcomas such as Kaposi's sarcoma.

EXAMPLE 1

Preparation of liposomal-all trans-retinoic acid (L-RA)

Preparation of lyophilized powder containing all trans-retinoic acid and phospholipids was carried out as follows. A solution of retinoic acid in t-butanol (1-5 mg/ml) was added to a dry lipid film containing dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) at a 7:3 molar ratio. The phospholipids were solubilized in the t-butanol containing the all-trans

retinoic acid and the solution was freeze-dried overnight. A powder containing dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), and all-trans retinoic acid was obtained. The lipid:drug ratio used was from 10:1 to 15:1.

Reconstitution of liposomal retinoic acid from the lyophilized powder was done as follows. The lyophilized powder was mixed with normal saline at room temperature to form multilamellar liposomes containing all trans-retinoic acid. This reconstitution method required mild hand-shaking for 1 min to obtain a preparation devoid of any aggregates or clumps. By light microscopy, the reconstituted preparation contained multilamellar liposomes of a close size range. No aggregates or drug clumps were identified in the liposomal preparation in three different experiments.

Encapsulation efficiency and size distribution of the liposomal all-trans retinoic acid preparation were determined as follows. The liposomal all-trans retinoic acid preparation was centrifuged at 30,000 x g for 45 minutes. A yellowish pellet containing the retinoic acid and the lipids was obtained. By light microscopy, the pellet was composed of liposomes with no crystals or drug aggregates. The encapsulation efficiency was calculated to be greater than 90% by measuring the amount of free retinoic acid in the supernatant by UV spectrophotometry. Liposomes were sized in a Coulter-Counter and Channelizer. The size distribution was as follows: 27% of liposomes less than 2 micrometers (um), 65% between 2 um and 3 um, 14% between 3 um and 5 um, 1% more than 5 um. The method used for encapsulation of retinoids was simple, reproducible and could be used for large scale production, for example, for clinical trials.

Further experiments were performed by the same procedure but with different lipids, ratios of lipids and the use of ^{3}H-all-trans retinoic acid. Additional lipids utilized were dipalmitoyl phosphatidyl choline (DPPC) stearylamine (SA) and cholesterol. After sedimentation of the liposomes, residual ^{3}H was determined and encapsulation efficiency calculated. Table 1 shows encapsulation efficiencies determined by this method for various L-RA preparations.

TABLE 1

Encapsulation Efficiency of
Retinoic Acid in Liposomes

LIPOSOME COMPOSITION	ENCAPSULATION EFFICIENCY (%)
DMPC:cholesterol 9:1	69.3
DMPC:cholesterol 9:3	64.5
DPPC	69.1
DMPC:SA:cholesterol 8:1:1	56.7
DMPC:DMPG 7:3	90
DMPC:DMPG 9:1	90.7

Of the lipid compositions studied, DMPC:DMPG at ratios between 7:3 and 9:1 gave superior encapsulation efficiencies. Liposomal all-trans retinol (L-ROH) was prepared by the methods described above for L-RA with DMPC:DMPG, 7:3.

EXAMPLE 2

Stability of Liposomal Retinoic Acid

Liposomal ^{3}H-retinoic acid (L-^{3}H-RA) was prepared with DMPC:DMPG, 7:3 as described in Example 1. Samples of the L-^{3}H-RA were incubated with either phosphate-buffered saline (PBS) or PBS with 20% (by volume) fetal calf serum (FCS). After various periods of incubation at about 37°C, aliquots were removed and centrifuged to sediment liposomes. The tritium in the supernatant solution was measured to determine ^{3}H-RA release. Figure 1 shows the release of ^{3}H-RA over a two day period. The L-^{3}H-RA was over about 80% stable over the period of the experiment, even in the presence of 20% FCS.

When ^{3}H-all-trans retinol was used to label L-ROH and stability in PBS measured, only about 5% of the ^{3}H-ROH was released after a 24 hr incubation at 37°C.

EXAMPLE 3

In Vitro Lysis of Human Erythrocytes (RBCs) by Retinoic Acid or Liposomal Retinoic Acid

Lysis of human red blood cells (RBCs) was quantitated by measuring the release of hemoglobin in the supernatants by observation of increases in optical density at 550 nanometers (nm), as described previously (Mehta, et al., Biochem. Biophys, Acta., Vol. 770-, pp 230-234 (1984). Free-RA dissolved in dimethyl formamide (DMFA), was added to the RBCs. Results with appropriate solvent controls, empty liposomes, and empty liposomes plus free-drug were also noted. Release of hemoglobin by hypotonic lysis of the same number of human RBCs by water was taken as a 100% positive control, while cells treated with PBS were taken as negative controls.

Preparations of L-RA comprising various lipids were incubated at a concentration of 20 microgram (μg) RA per ml with RBCs in PBS for 4 hr at 37°C. The toxicity of the L-RA preparations on the basis of percent RBC lysis is shown in Table 2.

TABLE 2

In Vitro Toxicity Of L-RA Preparations To RBCs

LIPOSOME COMPOSITION	% RBC LYSIS
DMPC:Cholesterol 9:1	4.5
DMPC:Cholesterol 9:3	90.2
DPPC	6.7
DMPC:SA:Cholesterol 8:1:1	70.4
DMPC:DMPG 7:3	8
DMPC:DMPG 9:1	8.3

As may be seen from the data of Table 2, L-RA of DMPC:cholesterol, DPPC, DMPC:DMPG (7:3) and DMPC:DMPG (9:1) exhibited low RBC toxicity under these conditions. It is of interest to note that the latter two L-RA compositions exhibited superior encapsulation efficiencies (Table 1).

A further experiment concerning the toxicity over time of free RA and L-RA (DMPC:DMPG-7:3) toward RBC was conducted. Human erythrocytes were incubated at 37°C in PBS with 10 ug/ml free RA or 120 ug/ml L-RA, and RBC lysis monitored over a period of 5 hr. Figure 2 shows time courses of RBC lysis. At between about 1 hr and about 3 hr, the free RA extensively lysed a large majority of the erythrocytes. When a similar manipulation was performed

with L-RA (DMPC:DMPG(7:3)) at a RA concentration of 120 ug/ml, little RBC lysis occurred (e.g., less than 10% after 6 hr).

A study was also conducted concerning the effects upon RBC lysis in 2 hr of free RA and L-RA (DMPC:DMPG(7:3)) at various concentrations. Figure 3 shows the results of this study. Free RA showed linearly increasing RBC lysis between about 5 ug RA/ml and about 30 ug RA/ml. Liposomal RA caused RBC lysis of only about 5% at a concentration of 160 ug RA/ml.

EXAMPLE 4

Acute Toxicity Of Free And Liposomal Retinoic Acid

The acute toxicity of free and liposomal all-trans retinoic acid was studied in CD1 mice. Free all-trans retinoic acid was prepared as an emulsion in normal saline containing 10% DMSO and 2% Tween 80 at a concentration of 3 to 5 mg/ml. Liposomal all-trans retinoic acid was prepared using a lipid:drug ratio of 15:1. The final concentration of all-trans retinoic acid in the liposomal preparation was 3 mg/ml. Empty liposomes of the same lipid composition (DMPC:DMPG 7:3) were also tested at doses equivalent to 80 mg/kg, 100 mg/kg, and 120 mg/kg of liposomal-all trans retinoic acid. Normal saline containing 10% DMSO and 2% Tween 80 was also tested as a control at a dose equivalent to 50 mg/kg of free all-trans retinoic acid. All drugs tested were injected intravenously via tail vein as a single bolus. The injected volumes of free and liposomal-all-trans retinoic acid were the same for each dose.

Table 3 shows data obtained from these acute toxicity experiments.

TABLE 3

Acute Toxicity of Free and Liposomal All-Trans Retinoic Acid

Drug	Dose (mg/kg)	Number Animals with seizures	Number Animals alive (72 hr)
Free RA	10	0/6	6/6
	20	6/6	5/6
	30	6/6	4/6
	40	3/3	0/3
	50	3/3	0/3
L-RA	40	0/6	6/6
	60	0/6	6/6
	80	0/6	6/6
	100	0/6	6/6
	120	0/6	6/6
Empty Liposomes	80	0/6	6/6
	100	0/6	5/6
	120	0/6	6/6
Normal saline 10% DMSO 2% Tween 80	50	0/6	6/6

The maximum non-toxic dose of free all-trans retinoic acid was 10 mg/kg. Higher doses caused seizures immediately after injection. The acute LD_{50} (deaths occurring up to 72 hours after injection) of free all-trans retinoic acid was 32 mg/kg. The cause of death was cardiopulmonary arrest after seizures for 1-2 minutes in all animals. No seizures or deaths were observed in the animals treated with liposomal all-trans retinoic acid at a dose of 120 mg/kg (maximum non-toxic dose and LD_{50} greater than 120 mg/kg). Higher doses were not tested. No seizures were observed in the animals treated with empty liposomes or normal saline with 10% DMSO and 2% Tween 80.

EXAMPLE 5

In Vitro Inhibition of Tumor Cell Growth

Liposomal all-trans retinoic acid (L-RA) was prepared as described in Example 1.

Cells of the human monocytic cell line THP-1 were inoculated into samples of eucaryotic cell culture medium in the presence or absence of L-RA, at a final RA concentration of 1 micromolar (um). After 24 hr at 37°C, ^{3}H-thymidine was added to each culture and incorporation thereof into cellular polynucleotides measured. Table 4 shows the percentage of tumor growth inhibition as reflected by decreases in ^{3}H-thymidine incorporation induced by L-RA of differing lipid compositions.

TABLE 4

L-RA Inhibition of Tumor Cell Growth

LIPOSOME COMPOSITION	TUMOR CELL (THP-1) INHIBITION (%)
DMPC:Cholesterol 9:1	72
DMPC:Cholesterol 9:3	22
DPPC	8
DMPC:SA:Cholesterol 8:1:1	84
DMPC:DMPG 7:3	70
DMPC:DMPG 9:1	32

From Table 4, it should be noted that L-RA (DMPC:DMPG-7:3), which, as previously shown herein, gave a superior

encapsulation efficiency and showed a low RBC toxicity (Tables 1 and 2), also effectively inhibited the tumor cell growth.

Cells of the human monocytic cell line THP-1 and of the human histiocytic cell line U-937 were inoculated at about 20,000 cells per cell in aliquots of eucaryotic cell culture medium contained in wells of a 96 well microtiter plate. The medium in various wells contained different amount of free RA or L-RA (DMPC:DMPG 7:3). The cells were incubated for 72 hr at 37°C and cell growth determined and compared to that of controls without any form of retinoic acid. Figure 4 shows the inhibition of THP-1 cell growth by increasing concentrations of free RA or L-RA (DMPC:DMPG 7:3). At concentrations of less than 1 μg RA/ml, both preparations inhibited cell growth by over 90%.

The human monocytic leukemia THP-1 cells, after a 72 hr incubation with either free RA or L-RA at a concentration of 0.3 μg RA/ml, were observed to have lost their generally ovate form and to have a more flattened and spread morphological appearance often associated with cellular differentiation. The generally ovate form was retained when the cells were cultured in the absence of any free or liposomal retinoic acid.

After incubation for 24 hr with 0.3 μg/ml or 0.6 μg/ml RA or L-RA in another experiment, THP-1 cells had increased levels of tissue transglutaminase enzymic activity, a marker for monocytic cell differentiation. As shown in Figure 5, THP-1 cells, at 4 x 10^6 cells/ml, showed about 50% greater transglutaminase activity when incubated with L-RA as compared to free RA at equivalent retinoic acid concentrations.

Cells of the human histiocytic cell line U-937 were distributed and cultured under the same conditions as the THP-1 cells in the prior experiment. Figure 6 shows the effects upon cell growth of increasing concentrations of free all-trans retinoic acid (RA), liposomal (DMPC:DMPG 7:3) all-trans retinoic acid (L-RA) and empty liposomes (which were devoid of retinoic acid). It should be noted that the U-937 cells were almost completely growth-inhibited by L-RA at a retinoic acid concentration of about 10 ug/ml while this amount of free RA inhibited growth less than 50%.

EXAMPLE 6

Antitumor Activity of Liposomal All-Trans Retinoic Acid in vivo

The antitumor activity of liposomal-all trans retinoic acid (DMPC:DMPG 7:3) was tested in vivo against liver metastases of M5076 reticulosarcoma. C57BL/6 mice were inoculated with 20,000 M5076 cells on day 0. Intravenous treatment with 60 mg/kg liposomal all-trans retinoic acid was given on day 4. The mean survival of control animals (non-treated) was 21.8 + 1.6 days. The mean survival of treated animals was 27.0 $\pm$ 1.6 days. Liposomal all-trans retinoic acid was shown, therefore, to have antitumor activity at a dose well below the maximum non-toxic dose, against a cell line (M5076) which was resistant to free retinoic acid in in vitro studies. THP-1 cells treated in vitro with RA (1 MM) for 72 hours when injected subcutaneously into male mice, failed to develop into tumors, whereas untreated cells formed a huge mass of tumors in such mice.

EXAMPLE 7

Induction of Tissue Transglutaminase
in Human Peripheral Blood Monocytes
by Intracellular Delivery of Retinoids

Circulating blood monocytes are the precursors of macrophages which accumulate at the sites of tumor rejection [2], delayed hypersensitivity [25], chronic inflammation [6], and at the site of damaged tissue as a part of the healing processes [11] (see reference citations in section D). At these sites, peripheral blood monocytes acquire new functional and biochemical characteristics that are associated with the maturation or differentiation process. To understand clearly the mechanisms involved in differentiation, it is necessary to manipulate the extracellular environment and assess precisely a variety of cellular functions and biochemical activities.

Vitamin A and its analogues (retinoids) have been shown to exert a profound effect on the differentiation of monocytic cells. Both normal [19] and leukemic [7,17,28] monocytic cells differentiate in response to retinoids which might suggest that retinoids play a role in regulating the differentiation of these cells. According to recent reports, the cellular activity of transglutaminase (TGase), an enzyme that catalyzes the covalent cross-linking of proteins, may be directly linked to the retinoid's action [4,15,21,23,35,39,39]. Recently, the present inventors found that <u>in vitro</u> maturation of human peripheral blood monocytes (HPBM) to macrophage-like cells was associated with the induction and accumulation of a specific intracellular TGase, tissue TGase [19,22]. Gamma (g)-interferon, which promotes the tumoricidal properties in HPBM, also augmented the expression of tissue TGase [19]. Similarly, the activation of guinea pig and mouse macrophages <u>in vivo</u> was associated with a marked increase in tissue TGase activity [10,24,34]. Terminal differen-

tiation of human monocytic leukemia cells (THP-1) induced by phorbol ester and retinoic acid was associated with induction and accumulation of tissue TGase (17], suggesting that the induction of tissue TGase was a marker of monocytic cell differentiation. The present invention involves further definition of the role of retinoids in differentiation and maturation of HPBM and comprises studies of culture conditions that inhibit or facilitate the internalization of retinoids by HPBM on expression of tissue TGase. The studies herein demonstrate that HPBM, isolated into two subpopulations, show no significant difference in their ability to express tissue TGase activity induced by either in vitro culture or exposure to recombinant interferon gamma (rIFN-g), and that the expression of tissue TGase in cultured HPBM may be induced by a direct delivery of retinoids to intracellular sites.

A. Materials and Methods

1. Materials

RPMI-1640 medium supplemented with L-glutamine and human AB serum were from Gibco Laboratories (Grand Island, NY); Escherichia coli-derived human recombinant g-interferon (rIFN-g) was kindly supplied by Genentech Inc. (South San Francisco, CA); and all-trans retinol (ROH) and all-trans retinoic acid (RA) were purchased from Sigma Chemical Co. (St. Louis, MO). The chromatographically pure lipids dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) were from Avanti Polar Lipids (Birmingham, AL); tritiated putrescine (sp. act. 28.8 Ci/mmol), from New England Nuclear (Boston, MA); and tritiated ROH (sp. act. 15 mci/mmol), from Amersham (Arlington Heights, IL). Lipids, culture medium, and serum were screened for endotoxin with the Limulus amebocyte lysate assay (MA Bioproducts, Walkersville,, MD), and they were used only when endotoxin contamination was less than 0.25 ng/ml.

2. HPBM Isolation, Purification, and Culture

Pure populations of HPBM were obtained by counter-current centrifugal elutriation of mononuclear leukocyte-rich fractions obtained from normal donors who were undergoing routine plateletapheresis [12]. HPBM were isolated into two subpopulations according to size with a Coulter ZBI counter and C-1000 channelizer (Coulter Electronics, Hialeah, FL). The median volume of small monocytes was 255 mm^3, and that of the large monocytes was 280 mm^3. The small monocytes were 95% ± 3% nonspecific esterase-positive and the large monocytes were 98% ± 2% positive. Detailed procedures for isolation and characteristics of these subpopulations have been published elsewhere [36,37]. Small, large, or mixed (obtained by mixing equal parts of small and large HPBM) HPBM subpopulations were washed once with medium (RPMI-1640 supplemented with L-glutamine, 20 mM HEPES buffer, 20 ug/ml gentamicin, and 5% human AB Serum) and resuspended to 0.5 million/ml density in the same medium. The cells were dispensed in 4-ml samples into 35-mm-well plates and cultured under appropriate conditions.

3. Enzyme Assay

Tissue TGase activity in cell extracts was measured as a Ca^{2+}, dependent incorporation of [^{3}H] putrescine into dimethylcasein. In brief, cultured HPBM were washed three times in Tris-buffered saline (20 mM Tris-HCl, 0.15 M NaCl, pH 7.6) and scraped from the dish in a minimal volume of the same buffer containing 1 mM EDTA and 15 mM Beta-mercaptoethanol. The cells were lysed by sonication, and TGase activity in the lysates was determined as described previously [13,20]. The protein content in cell lysates was determined by Lowry's method [14] with bovine gamma globulin as standard. The enzyme activity was expressed as nanomoles of putrescine incorporated into dimethyl-casein per hour per milligram of cell protein.

4. Immunochemical Detection of Tissue TGase

To detect tissue TGase in cell extracts, the cell lysates were solubilized in 20 mm Tris-HCl (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 0.75 M Beta-mercaptoethanol, 2.5% sucrose and 0.001% bromophenol blue. Solubilized extracts were fractionated by electrophoresis on a 6.5% discontinuous polyacrylamide gel and electro-blotted onto nitrocellulose paper. The paper was neutralized with 5% bovine serum albumin and treated with iodinated anti-tissue TGase antibody; the preparation, characterization and properties of this antibody have been described elsewhere (24]. The unbound antibody was removed by washing the paper in Tris-HCl buffer (50 mM, pH 7.5) containing 200 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.25% gelatin, and the paper was dried and autoradiographed as described earlier [20,24].

5. Preparation of Liposomes

Multilamellar vesicles (liposomes) containing DMPC and DMPG at a molar ratio of 7:3 were prepared as described [16,18]. All-trans ROH or RA were encapsulated by adding the required amount of the drug (predissolved in ethanol) in lipid-containing organic solvents before vacuum drying. The dried lipid-drug film was dispersed by agitation in sterile saline solution. Retinoids up to a 1:10 drug:lipid ratio could be completely encapsulated within the liposomes and were highly stable. The stability and encapsulation efficiency of the liposome preparations were studied by using radiolabelled retinol and showed that only 5% $\pm$ 2% of the incorporated radioactivity leaked out in the supernatant after 24-hr incubation at 37°C.

6. Binding Assay for [^{3}H]ROH

Freshly isolated HPBM were cultured in serum containing medium alone or medium plus 50 units (U)/ml rIFN-g for varying periods of time. At the end of

indicated time periods, HPBM monolayers were washed twice in ice cold medium and resuspended in 0.5 ml of prechilled reaction mixture containing 5.0 microcuries (μCi)/ml [11,12(n)^{3}H] vitamin A (free ROH) in RPMI medium supplemented with 5% delipidized human AB serum (serum delipidization was done by organic solvent extraction as described earlier [33]. Binding assays were carried out for 1 hr in an ice bath. After a 1-hr incubation, the monocyte monolayers were washed six times with ice-cold medium and the cells were lysed in 200 μl of Triton X-100. Fifty-microliter aliquots of cell lysates, in triplicate, were counted for the cell-associated radioactivity. Background counts, obtained by adding the reaction mixture toward the end of the 1-hr incubation before harvesting, were subtracted from the experimental values.

B. Results

1. Tissue TGase Induction During *In Vitro* Culture of HPBM

The culture of HPBM in the presence of serum-containing medium for up to 10 days was associated with a marked induction of tissue TGase activity in both small and large HPBM (Fig. 7), the increase in enzyme activity being more rapid after about 4 days of culture. After 10 days in culture, small monocytes showed a 93-fold increase in enzyme activity (from 0.44 to 41.1 nmol/hr/mg), whereas large HPBM accumulated about 103-fold increase in the enzyme activity (from 0.36 to 37.4 nmol/hr/mg). Small and large HPBM mixed together and cultured under similar conditions showed no significant difference in the rate and amount of accumulation of tissue TGase activity compared with that of individual HPBM fractions (data not shown). Induction of enzyme activity was associated with a change in the morphology of cultured monocytes. Freshly isolated HPBM looked rounded, but after 6-8 days in culture both the large and small HPBM became firmly adherent to the plastic

surface, were more spread and flattened, and had the appearance typical of mature macrophages. By day 10, when the cells had accumulated maximal levels of enzyme activity, these levels then either plateaued or started declining.

2. Effect of rIFN-g on Tissue TGase Expression

The effect of continuous exposure to rIFN-g on induction of tissue TGase activity in HPBM is shown in Figure 8. Small and large monocytes were cultured in serum-containing medium for 72 hr in the presence of increasing concentrations of rIFN-g. Enzyme activity in the HPBM populations increased significantly after their continuous exposure to rIFN-g compared with that of cells cultured in the presence of medium along. However, rIFN-g dose size produced no significant difference in enzyme activity between the two HPBM populations. As previously noted [19], a 100-U/ml dose of rIFN-g seemed to be optimal for augmenting TGase activity; higher rIFN-g-concentrations were less effective. The inductive effect of rIFN-g on tissue TGase activity was evidence at 5 U/ml and pretreatment of HPBM cultures with rIFN-g (100 U/ml) followed by washing and subsequent culture in medium alone did not enhance the expression of tissue TGase. The rIFN-g-induced augmentation of tissue TGase was associated with morphologic changes in HPBM so that the rIFN-g-treated cells were more spread out and flattened than the untreated control cells after three days in culture.

3. Effect of Retinoids on Tissue TGase Induction

Since the two HPBM populations showed no heterogeneity in terms of induced tissue TGase levels, our subsequent studies were done with whole HPBM fraction without separation into subsets. HPBM cultured in the presence of 500 nM RA for 24 hr accumulated at least three-fold higher

enzyme activity than did the control cells cultured in medium along (Fig. 9). Continuous exposure to RA caused a rapid and linear increase in the enzyme activity, whereas in the control cells no significant change in the level of tissue TGase activity was observed for up to 2 days of culture. By day 3, the control cells accumulated about six-fold higher enzyme activity (3.4 nmol/hr/mg) than did freshly isolated HPBM (0.6 nmol/hr/mg), but they still had significantly less enzyme activity than the RA-treated cells (9.8 nmol/hr/mg). Retinoic acid-induced expression of tissue TGase was dose dependent (Fig 9 inset). ROH, the physiologic analogue of RA, did not induce the expression of tissue TGase in HPBM even at a dose of 1 μM. Thus, HPBM cultured in the presence of ROH for up to 3 days showed no significant difference in accumulation of tissue TGase activity when compared with that of control cells cultured in medium along (Fig. 9).

4. Effect of Liposome-Encapsulated Retinoids on Tissue TGase Induction

Liposome-encapsulated RA was more effective in inducing tissue TGase expression than was free RA at an equimolar concentration. After 24-hr culture, the amount of tissue TGase activity in HPBM induced by free or liposomal RA at an equimolar concentration of 500 nM was not significantly different (3.4 and 3.7 nmol/hr/mg, respectively); after 48 and 72 hr, however, liposomal RA-treated cells accumulated at least 50% more enzyme activity than did free RA-treated cells (Fig. 10A). That increase in enzyme activity by liposome-encapsulated RA was a specific effect of RA and not of lipids was demonstrated by the fact that a culture of HPBM in the presence of "empty liposomes," and containing equivalent amount of lipids did not induce enzyme activity throughout the incubation period. "Empty liposomes," as reported earlier [20], inhibited serum-induced expression of tissue TGase after 72

hr of culture (Fig 10A). The free or liposomal RA-induced increase in enzyme activity was caused by an increased amount of the enzyme peptide, as revealed by Western-blot analysis of cell lysates using a iodinated antibody to tissue TGase (Fig. 10B). The increase in enzyme activity was proportional to the increase in enzyme peptide and not caused by activation of preexisting enzyme.

Retinol, which in its free form was unable to enhance the expression of tissue TGase in HPBM, became active when presented in liposomal form. Liposome-encapsulated ROH caused a rapid and linear increase in tissue TGase activity with time in culture (Fig 11A). After 72 hr of culture, liposomal-ROH caused a nine-fold increase in enzyme activity (7.1 nmol/hr/mg) when compared to that of control cells exposed to free ROH under similar conditions (0.8 nmol/hr/mg). Liposomal ROH-induced expression of tissue TGase resulted from increased accumulation of the enzyme peptide as demonstrated by Western-blot analysis (Fig. 11B).

5. Tissue TGase induction is Related to HPBM Uptake of Retinoids

The effect of <u>in vitro</u> maturation and rIFN-g treatment on the binding of tritiated-ROH by HPBM was examined. After 4 days of control culture (medium dose), tritiated-ROH binding by HPBM increased 50% compared to this binding by freshly isolated cells. After 9 days the control culture binding value increased to 350%. The increases in ROH binding were associated with parallel increases in tissue TGase activity (Table 5).

TABLE 5

Effect of In Vitro Culture and rIFN-g Treatment on [^{3}H]ROH Binding by HPBM

Culture Conditions	Days in Culture	[^{3}H]ROH bound (cpm/10 μg protein)	Tissue TGase activity (nmol/hr/mg)
medium alone	0	684 ± 25	0.25 ± 0.13
	4	994 ± 115	2.96 ± 0.75
	9	2,220 ± 144	32.60 ± 8.50
medium alone	3	626 ± 37	2.9 ± 0.23
medium + rIFN-g	3	1,782 ± 130	7.6 ± 0.7

[a] HPBM were cultured in serum-containing medium alone or medium containing 50 U/ml rIFN-g for indicated periods of time.

[b] Binding of tritiated ROH during different periods of culture was determined as described in Materials and Methods.

[c] Parallel cultures of HPBM maintained under similar conditions were used for assaying enzyme activity as described in Materials and Methods.

Exposure of HPBM to rIFN-g augmented the ROH binding and the expression of enzyme activity. The rIFN-g-treated cells showed a threefold higher [^{3}H]ROH binding than did control cells incubated in the presence of serum-containing medium alone for the same period of time. The presence of delipidized serum in the reaction mixture was essential; only 10% of the total counts were cell-associated when delipidized serum was omitted from the reaction mixture.

C. Discussion

The results reported in this Example suggested that HPBM, isolated into two populations based on their size and density, have equal potential to differentiate into mature macrophages. The in vitro maturation of HPBM to macrophages was associated with enhanced binding and uptake of retinol, presumably as a result of the acquisition of

cell surface receptors for serum retinol-binding protein. Exposure of HPBM to rIFN-g for 72 hr led to enhanced binding of [^{3}H]ROH that was comparable to the binding activity of control HPBM cultured in vitro for 9 days. HPBM maturation induced by in vitro culture or by exposure to rIFN-g was accompanied by similar morphologic and enzymatic changes. The requirement of cell surface receptor for serum retinol-binding protein could be circumvented by direct intracellular delivery of ROH.

Recently, several reports have suggested an association between monocytic cell differentiation and induction of tissue TGase [10,17,19,21-24,34]. Freshly isolated HPBM that have very low levels of tissue TGase accumulate large amounts of this enzyme after their in vitro maturation [19,22]. Just as the two subpopulations of HPBM showed no significant difference in their ability to induce and accumulate tissue TGase activity during in vitro differentiation to macrophages, both fractions were equally responsive to the effect of rIFN-g in terms of augmented enzyme expression (Fig. 8). Functional heterogeneity among HPBM subpopulations isolated by similar criteria has been reported earlier. Thus, the subsets of HPBM isolated into small and large populations have been reported to produce different amounts of reactive oxygen species [37], prostaglandins [1,30], antibody dependent cell-medicated cytotoxicity [27], and tumor-cell killing [26]. This functional heterogeneity among HPBM subpopulations has been attributed to either maturational or clonal differences. The data presented herein, however, suggest no heterogeneity among HPBM subpopulations in induction of tissue TGase, a marker for monocytic cell differentiation, and equal potential for differentiating into mature macrophag s. The ability of rIFN-g to enhance tissue TGase expression in both HPBM subpopulations suggests that this endogenous cytokine may play an

important role in the maturation, differentiation, and expression of differentiated functions in monocytic cells.

The factors in serum responsible for induction and accumulation of tissue TGase in cultured HPBM and macrophages have been shown to be endogenous retinoids and serum retinol-binding protein [21]. Extraction of retinoids by delipidization or depletion of retinol-binding protein from the serum completely abolished its enzyme-inducing ability [19,21]. Serum retinol-binding protein is believed to be responsible for intravascular transport and delivery of retinol to specific target tissues [8,9,29,31]. Receptors for serum retinol-binding protein present on the surface of target cells are responsible for the specificity of the delivery process [9,31]. The binding of ROH-retinol-binding protein complex to cell surface receptors apparently facilitates the delivery of ROH into the interior of the cell [9,31]. At superphysiologic doses (greater than 10 nM) on the other hand, RA can enter the cells directly by simple diffusion without the participation of surface receptors for retinol-binding protein [21]. This suggested that freshly isolated HPBM probably lack the cell surface receptors for serum retinol-binding protein and therefore cannot internalize the endogenous or exogenous retinoids. Indeed, the addition of exogenous RA to HPBM cultures at doses (e.g. greater than 10 nM) at which the receptor-mediated delivery becomes irrelevant resulted in a marked induction of tissue TGase activity (Fig. 9). The enzyme-inducing ability of RA was augmented further by encapsulating RA within the liposomes and allowing its internalization via phagocytosis (Fig. 10).

Of particular interest was the effect of ROH, which, in its free form did not induce the expression of tissue TGase in freshly isolated HPBM. When ROH was encapsulated

within liposomes, however, the requirement for a cell surface receptor for serum retinol-binding protein was obviated. Thus liposomal ROH induced a significant level of tissue TGase activity in HPBM (Fig. 11). This suggested an effective approach for targeting retinol or its inactive analogues to the monocytic cells with no or minimal toxic effects. Because HPBM lack cell surface receptors for serum retinol-binding protein makes administered ROH subject to nonspecific internalization by other cell types. The present studies suggested, furthermore, that interaction of ROH-retinol binding-protein complex with the cell surface receptor is required only for the intracellular delivery of retinol and that, unlike in the case of other hormones [3], ligand-receptor interaction may not require a second messenger for expression of the final event. The increase in TGase enzyme activity induced by free RA or liposome-encapsulated RA or ROH, was the result of the accumulation of enzyme protein rather than the activation of preexisting enzyme, as revealed by immunoblots of the cell lysates using an iodinated antibody to tissue TGase (Figs. 10,11).

Preliminary data on tritiated ROH-binding (Table 5) further supported the concept that <u>in vitro</u> differentiation of HPBM to mature macrophages was associated with acquisition of cell surface receptors for retinol-binding protein and that treatment with rIFN-g augmented the expression of these receptors. Once the HPBM acquire these receptors, they could internalize the endogenous retinoids and induce the expression of tissue TGase. Indeed, retinoids have been shown specifically to trigger the gene for tissue TGase in myelocytic cells [23].

Impairment of macrophage function in retinoid-deficient animals has been well documented to lead to increased incidence of infections and decreased tumor-cell

killing [5]. In cultures of guinea pig peritoneal macrophages, RA has been reported to increase the intracellular levels for the tumoricidal enzyme arginase [32]. The present findings that retinoids play an important role in the differentiation process of HPBM support the idea that retinoids are the important regulators of monocyte/macrophage functions.

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The preceding description is intended to illustrate specific embodiments of the present invention. It is not intended to be an exhaustive list of all possible embodiments. Person skilled in the relevant field will recognize that modifications could be made to the specific embodiments which have been disclosed, that would remain within the scope of the invention.

CLAIMS:

1. A carotenoid composition, comprising a carotenoid, lipid carrier particles, and an intercalation promoter agent; where the carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles, and where the composition is stable in an aqueous environment.

2. The composition of claim 1, where the carotenoid is substantially uniformly distributed in an intercalated position throughout a hydrophobic portion of the lipid carrier particles.

3. The composition of claim 1, where the molar ratio of carotenoid to lipid is greater than about 1:10.

4. The composition of claim 1, where the molar ratio of carotenoid to lipid is at least about 15:85.

5. The composition of claim 1, where the intercalation promoter agent comprises at least about 15% by weight of the composition.

6. The composition of claim 1, where the intercalation promoter agent is a triglyceride.

7. The composition of claim 1, where the lipid carrier particles are liposomes.

8. The composition of claim 7, where the liposomes are multilamellar.

9. The composition of claim 1, where the carotenoid is retinoic acid.

10. A retinoid composition, comprising a retinoid, liposomes, and a triglyceride; where the retinoid is substantially uniformly distributed with the lipid in the liposomes, where the molar ratio of retinoid to lipid is at least about 15:85, where the triglyceride is at least about 15% by weight of the composition, and where the composition is stable in an aqueous environment.

11. The composition of claim 10, where the retinoid is retinoic acid.

12. A composition comprising retinoic acid, liposomes whose lipid component consists essentially of dimyristoyl phosphatidyl choline, and a triglyceride; where the retinoic acid is substantially uniformly distributed with the dimyristoyl phosphatidyl choline in the liposomes, where the molar ratio of retinoic acid to dimyristoyl phosphatidyl choline is at least about 15:85, where the triglyceride is at least about 15% by weight of the composition, and where the composition is stable in an aqueous environment.

13. A pharmaceutical unit dosage formulation of a carotenoid, which comprises a carotenoid, lipid carrier particles, an intercalation promoter agent, and a pharmaceutically acceptable carrier; where the carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles, and where the formulation is stable in an aqueous environment.

14. The formulation of claim 13, where the ratio of lipid in the lipid carrier particles to the total liquid volume of the formulation is no greater than about 1 g:50 cc.

15. The formulation of claim 13, where the molar ratio of carotenoid to lipid in the lipid carrier particles is greater than about 1:10.

16. The formulation of claim 13, where the molar ratio of carotenoid to lipid is at least about 15:85.

17. The formulation of claim 13, where the intercalation promoter agent comprises at least about 15% by weight of the formulation, excluding the weight of the carrier.

18. The formulation of claim 13, where the intercalation promoter agent is a triglyceride.

19. The formulation of claim 13, where the formulation contains at least about 100 mg of carotenoid.

20. The formulation of claim 13, where the total liquid volume of the formulation is no greater than about 50 cc.

21. The formulation of claim 13, where the lipid carrier particles are liposomes.

22. The formulation of claim 21, where the liposomes are multilamellar.

23. The formulation of claim 13, where the carotenoid is retinoic acid.

24. A pharmaceutical unit dosage formulation of a retinoid, which comprises a retinoid, liposomes, and a triglyceride; where the retinoid is substantially uniformly distributed with the lipid in the liposomes, where the molar ratio of retinoid to lipid is at least about 15:85, where the triglyceride is at least about 15% by weight of

the composition, and where the composition is stable in an aqueous environment.

25. The formulation of claim 24, where the retinoid is retinoic acid.

26. A pharmaceutical unit dosage formulation of retinoic acid, which comprises retinoic acid, liposomes whose lipid component consists essentially of dimyristoyl phosphatidyl choline, and a triglyceride; where the retinoic acid is substantially uniformly distributed with the dimyristoyl phosphatidyl choline in the liposomes, where the molar ratio of retinoic acid to dimyristoyl phosphatidyl choline is at least about 15:85, where the triglyceride is at least about 15% by weight of the composition, and where the composition is stable in an aqueous environment.

27. A method of inhibiting the growth of cancer cells, comprising administering to a living subject a therapeutically effective amount of a carotenoid composition which comprises a carotenoid, lipid carrier particles, and an intercalation promoter agent; where the carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles, and where the composition is stable in an aqueous environment.

28. The method of claim 27, where the composition is administered to the subject in a maintained carotenoid:lipid molar ratio between about 5:85 and 15:70, and where the composition has reduced toxicity to normal cells as compared to free carotenoid.

29. The method of claim 27, where the carotenoid is substantially uniformly distributed in an intercalated position throughout a hydrophobic portion of the lipid carrier particles.

30. The method of claim 27, where the molar ratio of carotenoid to lipid is greater than about 1:10.

31. The method of claim 27, where the molar ratio of carotenoid to lipid is at least about 15:85.

32. The method of claim 27, where the intercalation promoter agent comprises at least about 15% by weight of the composition.

33. The method of claim 27, where the intercalation promoter agent is a triglyceride.

34. The method of claim 27, where the lipid carrier particles are liposomes.

35. The method of claim 34, where the liposomes are multilamellar.

36. The method of claim 27, where the carotenoid is retinoic acid.

37. A method of inhibiting the growth of cancer cells, comprising administering to a living subject a therapeutically effective amount of a retinoid composition which comprises a retinoid, liposomes, and a triglyceride; where the retinoid is substantially uniformly distributed with the lipid in the liposomes, where the molar ratio of retinoid to lipid is at least about 15:85, where the triglyceride is at least about 15% by weight of the composition, and where the composition is stable in an aqueous environment.

38. The method of claim 37, where the retinoid is retinoic acid.

39. A method of inhibiting the growth of cancer cells, comprising administering to a living subject a therapeutically effective amount of a retinoic composition which comprises retinoic acid, liposomes whose lipid component consists essentially of dimyristoyl phosphatidyl choline, and a triglyceride; where the retinoic acid is substantially uniformly distributed with the dimyristoyl phosphatidyl choline in the liposomes, where the molar ratio of retinoic acid to dimyristoyl phosphatidyl choline is at least about 15:85, where the triglyceride is at least about 15% by weight of the composition, and where the composition is stable in an aqueous environment.

STABILITY OF ^{3}H-RETINOIC ACID IN PRESENCE OR ABSENCE OF SERUM PROTEINS AFTER ENCAPSULATION IN LIPOSOMES.

Fig. 1

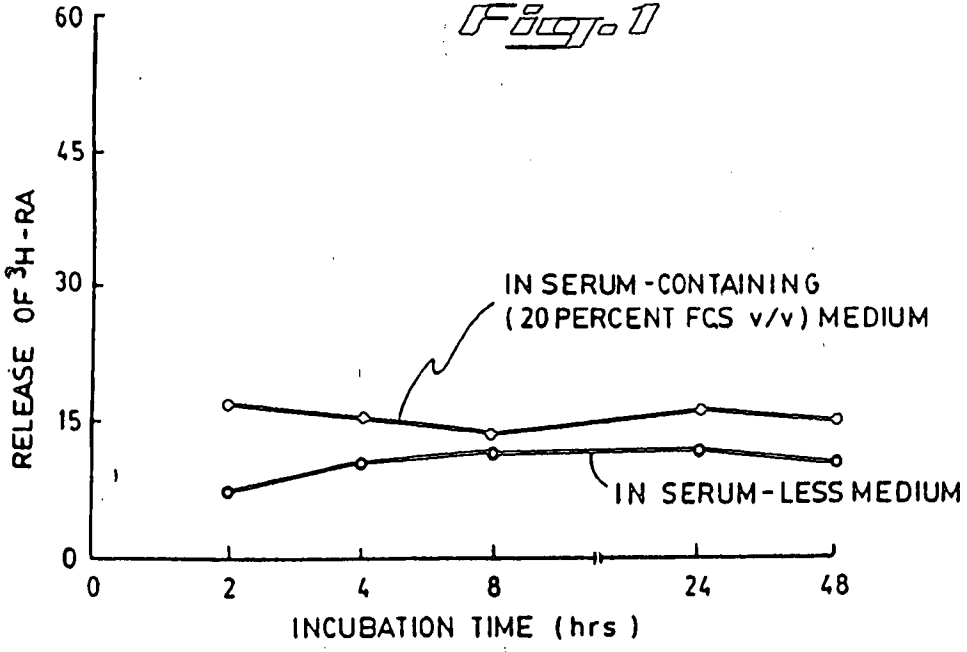
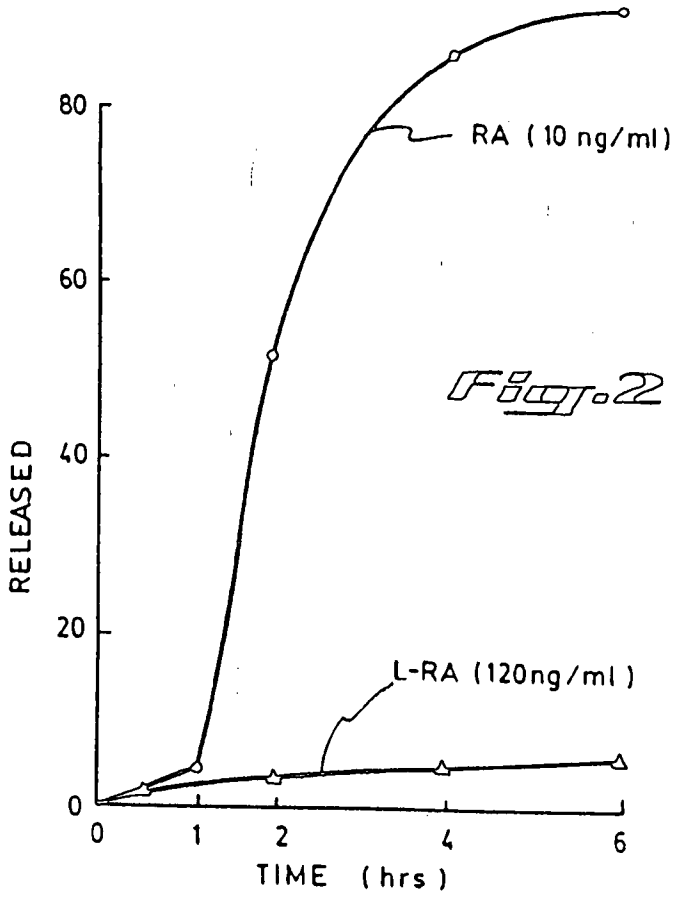


Fig. 2



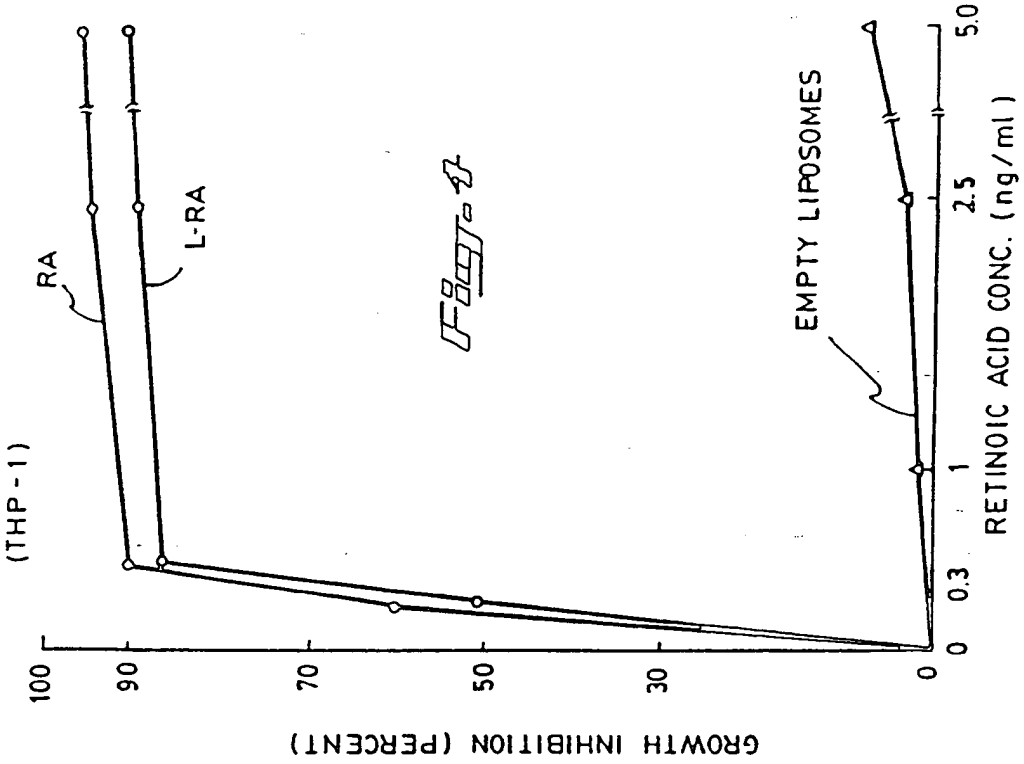


Fig. 4

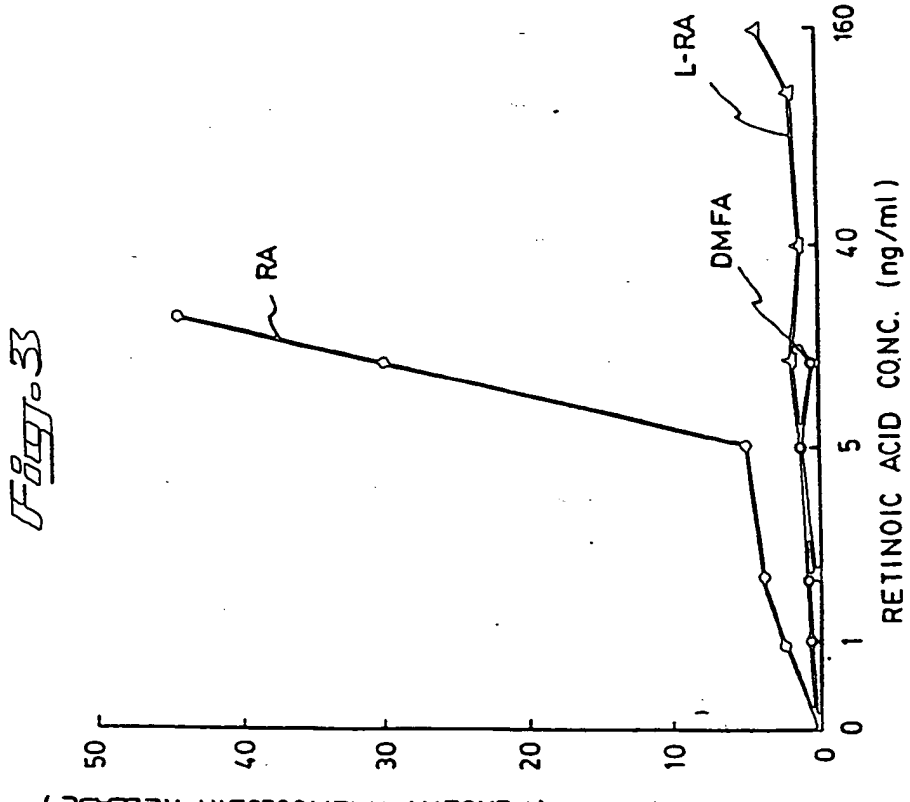


Fig. 3

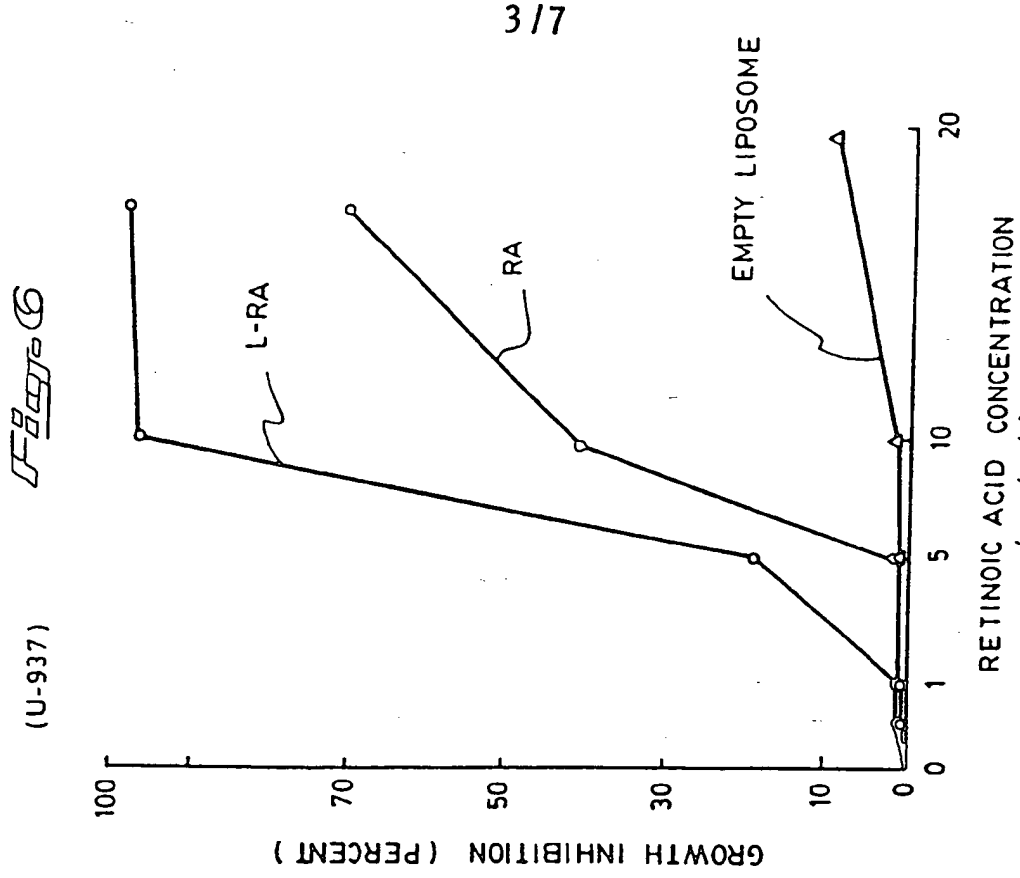


Fig. 6

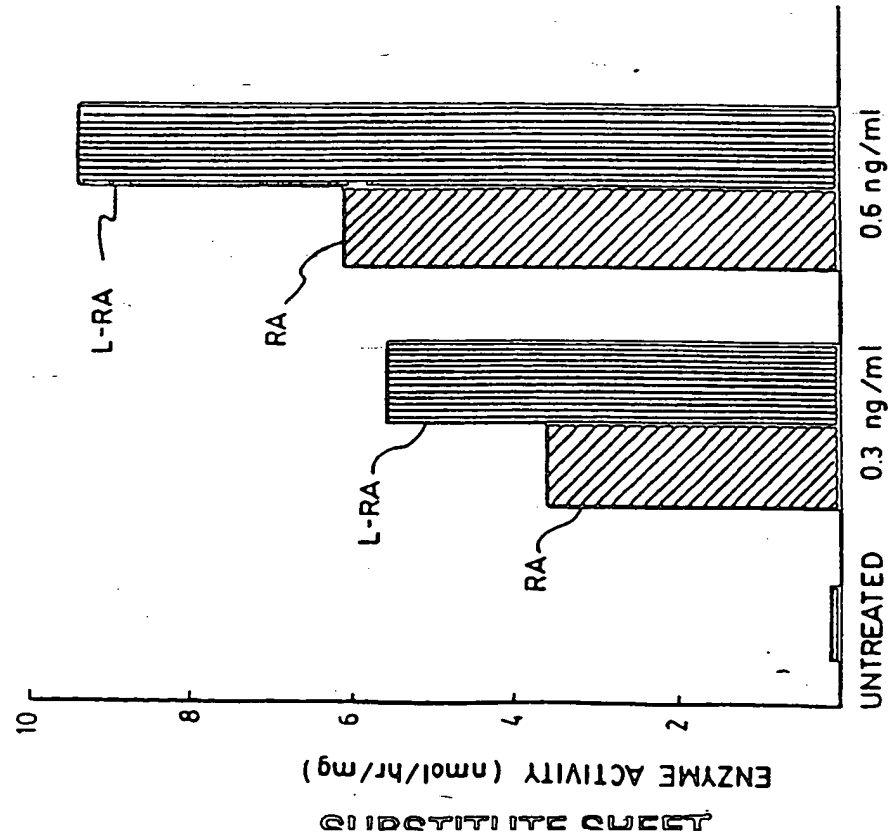


Fig. 5

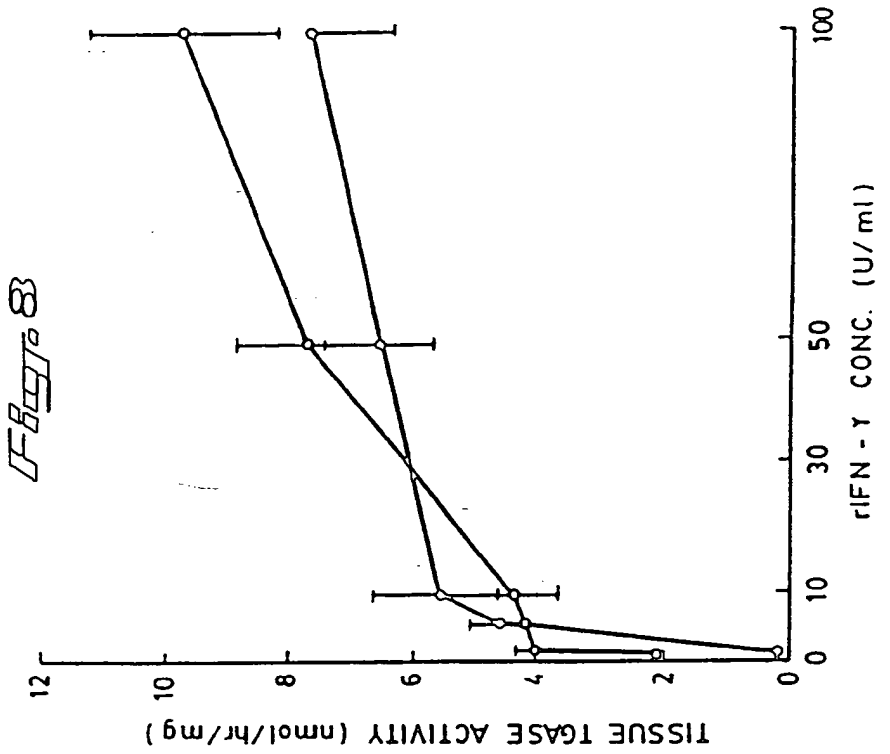


Fig. 8

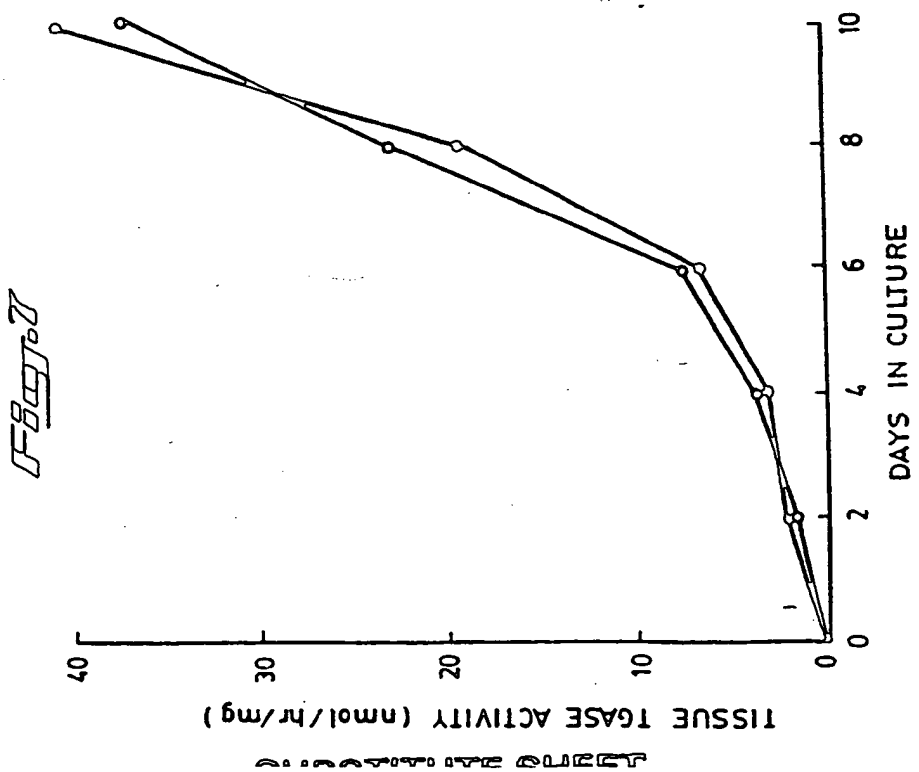


Fig. 7

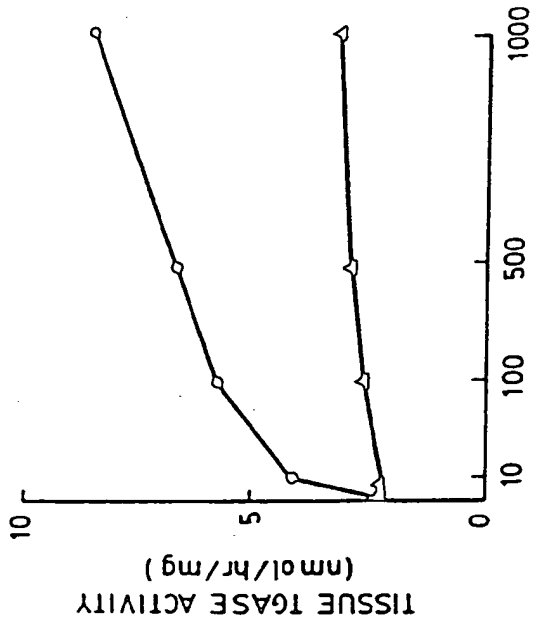


Fig. 9B

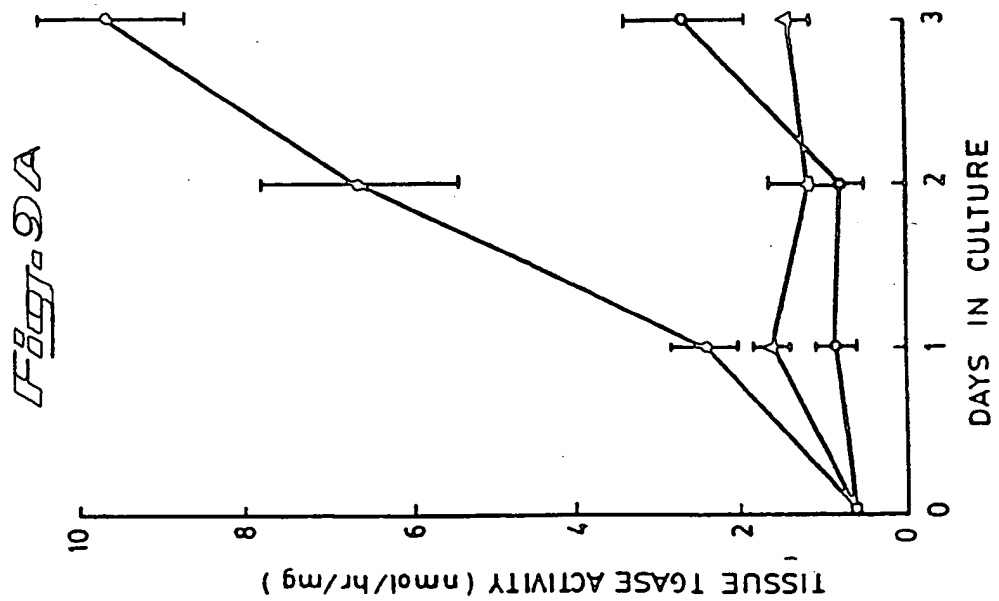
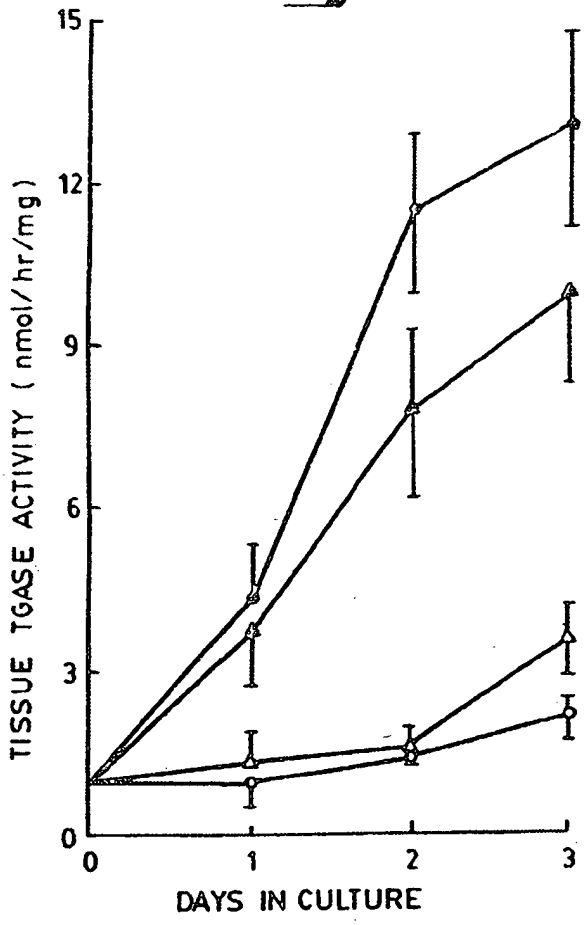


Fig. 9A

Fig. 10A



1 2 3 4 5

Fig. 10B

Fig. 11A

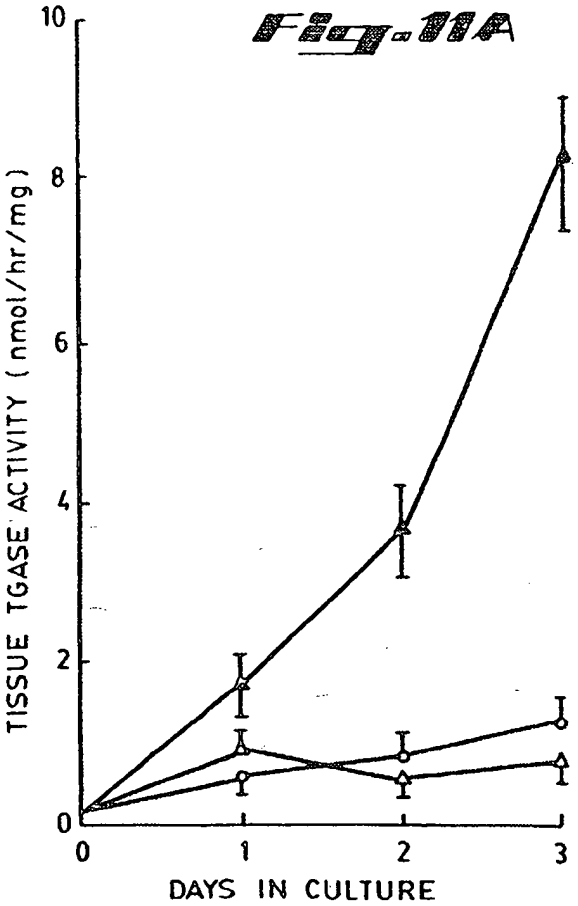


Fig. 11B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/00233

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K9/127; A61K31/20

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,8 911 850 (FOUNTAIN) 14 December 1989 see page 3, line 1 - line 22 see page 5, line 15 - line 21 see page 40; example 63	1-26
X	FR,A,2 455 458 (KUREHA KAGAKU KOGYO KK) 28 November 1980 see the whole document see page 7, line 6 - line 16	1-8
X Y	WO,A,9 112 794 (NATTERMANN, A & CIE. GMBH) 5 September 1991 see page 2, line 5 - line 14 see page 3, line 6 - line 9 see page 4, line 7 - line 12 see page 7, line 19 - page 8, line 10	1-8,10, 13-22,24 1-39

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 APRIL 1993	12.05.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BENZ K.F.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 906 977 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 10 August 1989 see the whole document ---	1-39
X	EP,A,0 287 198 (BOARD OF REGENTS THE UNIVERSITY OF TEXAS SYSTEM) 19 October 1988 see page 3, line 26 - page 5, line 21 see page 13, line 34 - line 51 see page 19, line 1 - line 46 -----	1,2

..ernational application No.

INTERNATIONAL SEARCH REPORT

PCT/US 93/00233

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

ALTHOUGH CLAIMS 27-39 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN/AN IMAL BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOSITION.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9300233
SA 69291

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/04/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8911850	14-12-89	AU-A- 2719492	07-01-93
		AU-B- 628355	17-09-92
		AU-A- 3777789	05-01-90
		EP-A- 0372070	13-06-90
		JP-T- 2504642	27-12-90
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		JP-A- 63301824	08-12-88
		US-A- 5043329	27-08-91
		US-A- 4950656	21-08-90
		US-A- 5032585	16-07-91

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82